

Clinical Efficacy and Predictive Molecular Markers of Neoadjuvant Gemcitabine and Pemetrexed in Resectable Non-small Cell Lung Cancer

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Background: A trial of neoadjuvant gemcitabine and pemetrexed (GP) chemotherapy in patients with resectable non-small cell lung cancer was conducted. The goal was to achieve a disease response rate of 50% and to determine if the expression levels of genes associated with GP metabolism are predictive of response.

Methods: Patients had staging with a computed tomography scan, whole body F-18 fluorodeoxyglucose positron emission tomography, and mediastinoscopy. Four biweekly cycles of GP were given. Patients were restaged, and those with resectable stage IB-III disease had thoracotomy. Fresh frozen tumor specimens were collected before and after chemotherapy and the mRNA levels of 14 target genes determined by real-time reverse transcription polymerase chain reaction.

Results: Fifty-two patients started therapy. The radiographic disease response rate was 35% (95% confidence interval 21.7–49.6%), and the progression rate was 6%. Forty-six patients had a thoracotomy. The complete tumor resection rate was 77% (40/52). There were no perioperative deaths or deaths related to chemotherapy. Tumor response to chemotherapy was inversely correlated with the level of expression of *RRM1* ($p < 0.001$; regulatory subunit of ribonucleotide reductase) and *TS* ($p = 0.006$; thymidylate synthase); i.e., the reduction in tumor size was greater in those with low levels of expression.

Conclusions: Neoadjuvant GP is well tolerated and produces an objective response rate of 35%. Tumoral *RRM1* and *TS* mRNA levels are predictive of disease response and should be considered as parameters for treatment selection in future trials with this regimen.

Key Words: Gemcitabine, Pemetrexed, Ribonucleotide reductase, Thymidylate synthase, Non-small cell lung cancer.

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Incorporation of platinum-containing chemotherapy into the management of resectable non-small cell lung cancer (NSCLC) has become the standard of care for patients with metastatic disease in N1 or N2 lymph nodes.^{1–3} Neoadjuvant treatment results in response rates of approximately 33 to 64%,^{4–7} and adjuvant therapy increases absolute overall survival (OS) by approximately 5 to 15%.^{1–3} However, the approach of treating all patients with a platinum-containing regimen may have reached a plateau in terms of efficacy. In addition, there is significant toxicity associated with this approach including a treatment-related mortality of approximately 1 to 2%.^{1,7}

We had reported a response rate of 34% in patients with operable stage I–III disease using a nonplatinum regimen consisting of gemcitabine and vinorelbine in a phase II neoadjuvant trial.⁸ The combination of gemcitabine and pemetrexed (GP) had yielded a response rate of 31% in patients with previously untreated advanced-stage NSCLC if given on a 3 weekly schedule, and efficacy seemed to be dependent on the drug sequencing and scheduling.⁹ However, prior investigations of both drugs suggested that synergy as a result of sequencing may vary from one in vitro system to another.^{10–12} In a phase I trial of G immediately followed by P given biweekly, the maximum tolerated doses were 1500 and 500 mg/m², respectively.¹³ The regimen produced a response rate of 21% in untreated patients with advanced NSCLC, and it was well tolerated.¹³

We conducted a single-institution trial of neoadjuvant GP in patients with resectable NSCLC with the goal to describe the clinical efficacy and tolerability of the chosen regimen and to investigate the predictive utility of mRNA expression of genes involved in the metabolism of these drugs on therapeutic efficacy.

PATIENTS AND METHODS

Clinical Trial Characteristics and Study Population

The study was approved by the University of South Florida's Institutional Review Board (ClinicalTrials.gov NCT00226577). Clinical staging was determined by physical

TABLE 1. mRNA Gene Expression Characteristics and Association with Radiographic Disease Response

	Gene Name	Probe set ID	N	Min.	Max.	Median	Mean	Spearman's Rho	p
Clinical Response			49	95% (increase)	−100% (decrease)	−19%	−19%		
RRM1	Regulatory subunit of ribonucleotide reductase	— ^a	35	0.37	7.93	1.68	2.72	0.649	≤0.001
RRM2a	Catalytic subunit of ribonucleotide reductase	Hs00367247-m1	32	0.00	5.42	0.90	1.38	−0.017	0.924
RRM2b	P53-inducible catalytic subunit of ribonucleotide reductase	Hs00153082-m1	32	0.00	12.24	1.34	1.99	0.276	0.125
DCK	Deoxycytidine kinase	Hs00176127-m1	32	0.46	40.39	3.82	5.52	0.130	0.476
CDA	Cytidine deaminase	Hs00156401-m1	32	0.00	118.66	2.42	10.27	0.028	0.878
ENT1 (SLC29A1)	Equilibration sensitive nucleoside transporter 1	Hs00191940-m1	32	0.00	21.42	1.30	2.18	0.162	0.374
5'-NT (NT5C1A)	Cytosolic 5'-nucleotidase	Hs00261369-m1	32	0.00	170843.81	0.00	10.89	−0.081	0.657
TS	Thymidylate synthase	Hs00426591-m1	35	0.32	18.31	3.39	4.46	0.454	0.006
DHFR	Dihydrofolate reductase	Hs00758822-s1	32	0.00	9.83	0.85	1.29	0.166	0.362
GARFT	Phosphoribosylglycinamide formyl transferase	Hs00531926-m1	32	0.17	21.93	1.19	2.28	0.002	0.988
FPGS	Folypolyglutamate synthase	Hs00191956-m1	32	0.36	32.75	2.23	3.50	0.222	0.220
ENT2 (SLC29A2)	Equilibration sensitive nucleoside transporter 2	Hs00155426-m1	32	0.11	41.30	5.16	7.76	0.125	0.494
RFC1 (SLC19A1)	Reduced folate carrier	Hs00161870-m1	32	0.01	33.47	0.12	2.23	0.096	0.600
γ-GH	Gamma-glutamyl hydrolase	Hs00608257-m1	32	0.07	33.52	2.47	5.75	−0.015	0.934

^a 5'-FAM-TTTGC TCTTT GGATT CCGGA TCTCT TCA-TAMRA-3'.
Min., minimum; max., maximum.

examination, computed tomography (CT) of the chest and upper abdomen, whole body F-18 fluorodeoxyglucose positron emission tomography, magnetic resonance imaging of the brain, bronchoscopy, and mediastinoscopy. Histologic confirmation of NSCLC; stage IB-IIIa and selected IIIB (two lesions in one lobe, T4); age ≥18 years; a performance status (PS) of 0 to 1; measurable disease by Response Evaluation Criteria in Solid Tumors; and no prior therapy for lung cancer were required for eligibility. These criteria were met by 52 patients.

Preoperative chemotherapy with G 1500 mg/m² immediately followed by P 500 mg/m² was administered on days 1, 15, 29, and 43. Subsequent doses of chemotherapy were delayed or reduced for toxicity if appropriate. Patients received oral folate at a dose of 350 to 1000 μg daily and subcutaneous vitamin B12 at a dose of 1000 μg every 9 weeks starting 1 week before chemotherapy. Dexamethasone

was given at a dose of 4 mg every 12 hours on the day before, the day of, and the day after chemotherapy. All toxicities were graded according to the common toxicity criteria (version 3.0).

After chemotherapy, CT and positron emission tomography scans were repeated between days 50 and 63. Radiographic response was expressed as a continuous variable by calculating the percentage of change in the sum of all greatest tumor diameters comparing the posttreatment and pretreatment CT scans (1 − [sum postlesions/sum prelesions] × 100) and also by Response Evaluation Criteria in Solid Tumors as best overall response.

Patients with resectable disease had thoracotomy between days 64 and 77. The recommended surgery was lobectomy or pneumonectomy with mediastinal lymph node dissection. Segmentectomy or wedge resection was discouraged. Patients with unresectable disease and those with incomplete

TABLE 2. Patient Characteristics, Disease Response, and Survival

		Radiographic Response Rate ^a		Pathologic Response Rate ^b		Median Overall Survival	Median Disease-Free Survival ^c	
All patients	N = 52	35%	(17/49)	30%	(13/43)	>27.8 mo	33.7 mo	
Age (yr), median (range)	67 (41–83)							
<67	n = 26	38%	(9/24)	25%	(6/24)	>27.0 mo	>21.1 mo	
≥67	n = 26	32%	(8/25)	33%	(7/21)	>27.8 mo	21.0 mo	p = 0.45
Gender								
Women	n = 26	32%	(8/25)	27%	(6/22)	>27.8 mo	21.1 mo	
Men	n = 26	38%	(9/25)	33%	(7/21)	>27.4 mo	33.7 mo	p = 0.75
Performance status								
Zero	n = 31	36%	(10/28)	36%	(10/28)	>27.8 mo	33.7 mo	
One	n = 21	33%	(7/21)	20%	(3/15)	16.8 mo	>11.6 mo	p = 0.96
Weight loss ^d								
Absent	n = 47	34%	(15/44)	31%	(12/39)	>27.4 mo	33.7 mo	
Present	n = 5	40%	(2/5)	25%	(1/4)	16.1 mo	9.1 mo	p = 0.31
Smoking status ^e								
Active	n = 23	30%	(6/20)	21%	(4/19)	>25.0 mo	21.0 mo	
Quit (>1 yr)	n = 27	41%	(11/27)	36%	(8/22)	>27.8 mo	>20.7 mo	p = 0.37
Never smoker	n = 2	NA	(0/2)	NA	(1/2)			
Histopathology ^f								
Squamous	n = 19	39%	(7/18)	22%	(4/18)	>27.8 mo	>20.7 mo	
Nonsquamous	n = 33	32%	(10/31)	36%	(9/25)	>27.4 mo	21.1 mo	p = 0.73
Stage								
I	n = 16	31%	(5/16)	27%	(4/15)	>27.8 mo	20.7 mo	
II	n = 18	38%	(6/16)	29%	(4/14)	>19.8 mo	20.8 mo	p = 0.33
III ^g	n = 18	35%	(6/17)	36%	(5/14)	>25.0 mo	>21.1 mo	
Tumor resection								
Complete	n = 40	37%	(14/38)	33%	(13/39)	>27.8 mo	33.7 mo	
Incomp./not done	n = 12	27%	(3/11)	0%	(0/4)	12.8 mo	NA	p = NA
Response to chemotherapy ^h								
CR/PR	n = 17	NA	(17/17)	38%	(6/16)	>27.4 mo		
SD	n = 29	NA	(0/29)	24%	(6/25)	>27.8 mo	18.2 mo	
PD	n = 3	NA	(0/3)	0%	(0/1)	>28.0 mo	>33.7 mo	p = 0.39

^a Not assessed in three patients.^b Not assessed in nine patients.^c In the 40 patients with complete tumor resection.^d Equal to or greater than 5% during the 3 months before diagnosis.^e A life-time never smoker was defined as a person that had smoked less than a total of 100 cigarettes.^f Twenty-one patients had adenocarcinoma including five with bronchioloalveolar features, 12 had other NSCLC subtypes including two adenosquamous carcinomas, two mixed adenocarcinoma and squamous carcinoma, and one large cell carcinoma with suggestive neuroendocrine features.^g Two T4N0, T4 because of two separate tumor nodules in a single lobe of the lung.^h The pathologic disease response categories pPR and pNR were not significantly associated with OS (hazard ratio for pPR versus pNR = 1.1, *p* = 0.90) or DFS (hazard ratio for pPR versus pNR = 0.87, *p* = 0.78).

NA, not applicable; incomp., incomplete; CR, complete remission; PR, partial remission; SD, stable disease; PD, partial disease.

resections were treated at the discretion of their physician. All patients were followed at 3 month intervals for 2 years and then every 6 months with a CT scan.

Molecular Investigations

Tumor samples were collected before and after therapy as frozen specimens. The standard operating procedure for collection included a recording of the time from biopsy or resection to freezing, and the time elapsed was 30 minutes or less in all cases. Frozen specimens were embedded in optimal cutting temperature medium and cut in 5 to 7 μ m sections.

Tumor cells were collected by laser capture microdissection using the Arcturus system. Total RNA was extracted using

a commercial method (Arcturus, Mountain View, CA), and cDNA was generated with oligo-dT and random primers. Real-time quantitative polymerase chain reaction analysis was performed in triplicate per sample (7900HT, ABI, Foster City, CA). The probe and primers for *RRM1* were those previously described.¹⁴ Commercially available primers and probes were used for expression analysis of all other target genes (Table 1). The relative amount of *RRM1* and *TS* mRNA in a sample was determined by comparing the threshold cycle with a standard curve as described.¹⁴ For the remaining genes, relative quantification was performed by comparison of the test samples to a single calibrator sample in a fluidic card assay. Negative controls without a cDNA template were included in all experiments.

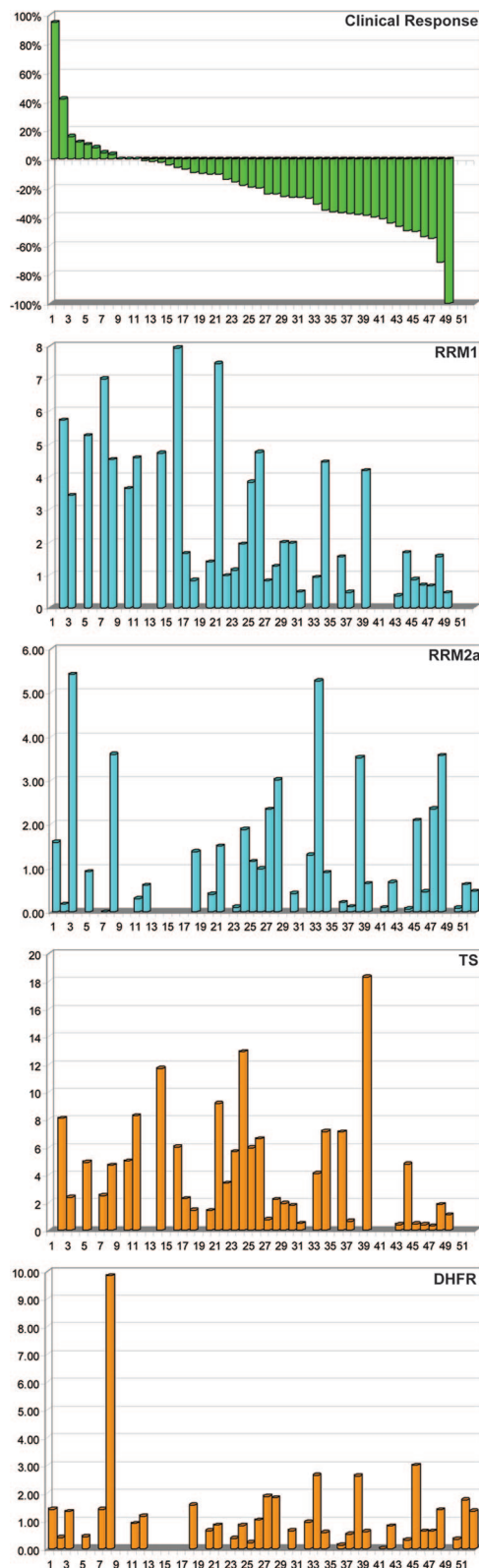


FIGURE 1. Water fall plots of radiographic disease response and mRNA gene expression.

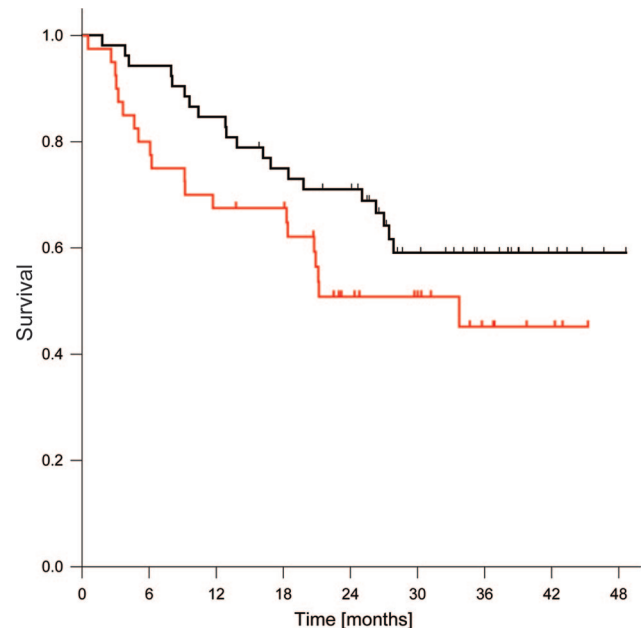


FIGURE 2. Kaplan-Meier overall and disease-free survival (OS and DFS) estimates. The black curve denotes OS and the red curve DFS. Tick marks indicate censored cases.

Statistical Considerations

Correlation coefficients between the expression of 14 genes, seven each potentially related to G and P efficacy, and the continuous variable tumor response were calculated according to Spearman.¹⁵ The statistical significance level was adjusted for multiple analyses according to Bonferroni to a *p* value of 0.0036; i.e., 0.05 divided by 14, because 14 independent or dependent genes were tested on the same dataset.¹⁶ The two-sided pooled *t* test or Wilcoxon Rank Sum test was used to test for significance between dichotomous variables and gene expression. The Kruskal Wallis test was used to test for significance between noncontinuous variables with more than two values and gene expression. Kaplan-Meier survival estimates were generated to describe the disease-free survival (DFS) and OS. OS was estimated from the date of diagnosis to the date of death or last observation, and DFS was estimated from the date of surgery to the date of recurrence, death, or last observation. Patients without an event were censored as of the date of last observation.

RESULTS

Induction Chemotherapy and Toxicity

A total of 52 eligible patients, 26 men and 26 women, between the ages 41 and 83 years (median 67 years), received at least one dose of chemotherapy. They were enrolled between April 2004 and April 2006, and their characteristics are described in Table 2. Forty-two patients received all planned chemotherapy on time and without dose reduction. Three patients had dose delays without dose reductions. The reasons were a grade 3 lower extremity cellulitis, a grade 2 thrombocytopenia, and a grade 3 liver enzyme elevation. Seven patients had less than the intended four biweekly

therapies. Three patients had only the first cycle because of grade 3 neutropenic fever with renal failure (stage T2N2M0), a grade 3 febrile drug reaction, and one patient requested immediate surgery without having experienced treatment-related side effects. Three patients only had cycles 1 and 2. The reasons were a grade 3 lower extremity phlebitis in one patient and grade 3 fatigue in two. One patient (stage T2N2M0) died after cycle 3 from pneumonitis of a likely viral etiology; however, a possibly treatment-related etiology could not be excluded.

Radiographic Response to Chemotherapy

A radiographic response evaluation was possible in 49 patients. The best overall response was a complete remission (CR) in 1 (2%; 95% confidence interval [CI] 0.1–10.9%) patient; it was a partial remission (PR) in 16 (33%; 95% CI 20.0–47.5%), stable disease in 29 (59%; 95% CI 44.2–73.0%), and progressive disease in 3 (6%; 95% CI 1.3–16.9%) patients. The response ranged from a 95% increase to a 100% decrease in the size of measurable lesions (Figure 1A). There were no statistically significant associations between radiographic disease response and the clinical parameters age ($p = 0.62$), gender ($p = 0.96$), PS ($p = 0.94$), weight loss ($p = 0.88$), smoking status ($p = 0.85$), tumor histology ($p = 0.95$), and stage ($p = 0.31$) (Table 2).

Pathologic Response to Chemotherapy

A pathologic response evaluation was performed by determination of the proportion of necrotic and/or fibrotic material on light microscopical evaluation of surgical resection specimens stained with hematoxylin and eosin. This was possible in 43 patients, and it ranged from 0 to 90%. None of the patients had a pathologic CR ($\geq 95\%$ necrosis/fibrosis), 13 (30%) had a pathologic PR (50–94% necrosis/fibrosis), and 30 (70%) had no pathologic response. There were no statistically significant associations between pathologic disease response and the clinical parameters age ($p = 0.39$), gender ($p = 0.65$), PS ($p = 0.18$), weight loss ($p = 0.69$), smoking status ($p = 0.96$), tumor histology ($p = 0.43$), and stage ($p = 0.66$). Although there was a correlation between radiographic and pathologic response (Spearman's $\rho = 0.23$); i.e., better clinical response was associated with a higher proportion of necrosis and fibrosis, it was not statistically significant ($p = 0.14$).

Surgical Treatment

A thoracotomy was performed in 46 patients, and it resulted in a complete resection in 40 patients. Thirty-two patients had a lobectomy, two had a bilobectomy, eight had a pneumonectomy, two had a wedge resection, and two had no tumor resection. At the surgical resection, 17 patients were down-staged, 17 had no change, and 12 were up-staged compared with the initial staging. One patient died 2.7 months after a complete right upper and middle lobectomy for a T2N1 squamous cell carcinoma of a bronchopleural fistula.

Recurrence and Survival

As of the index date June 20, 2008, 32 patients were alive (20 without evidence for disease, seven with recurrence,

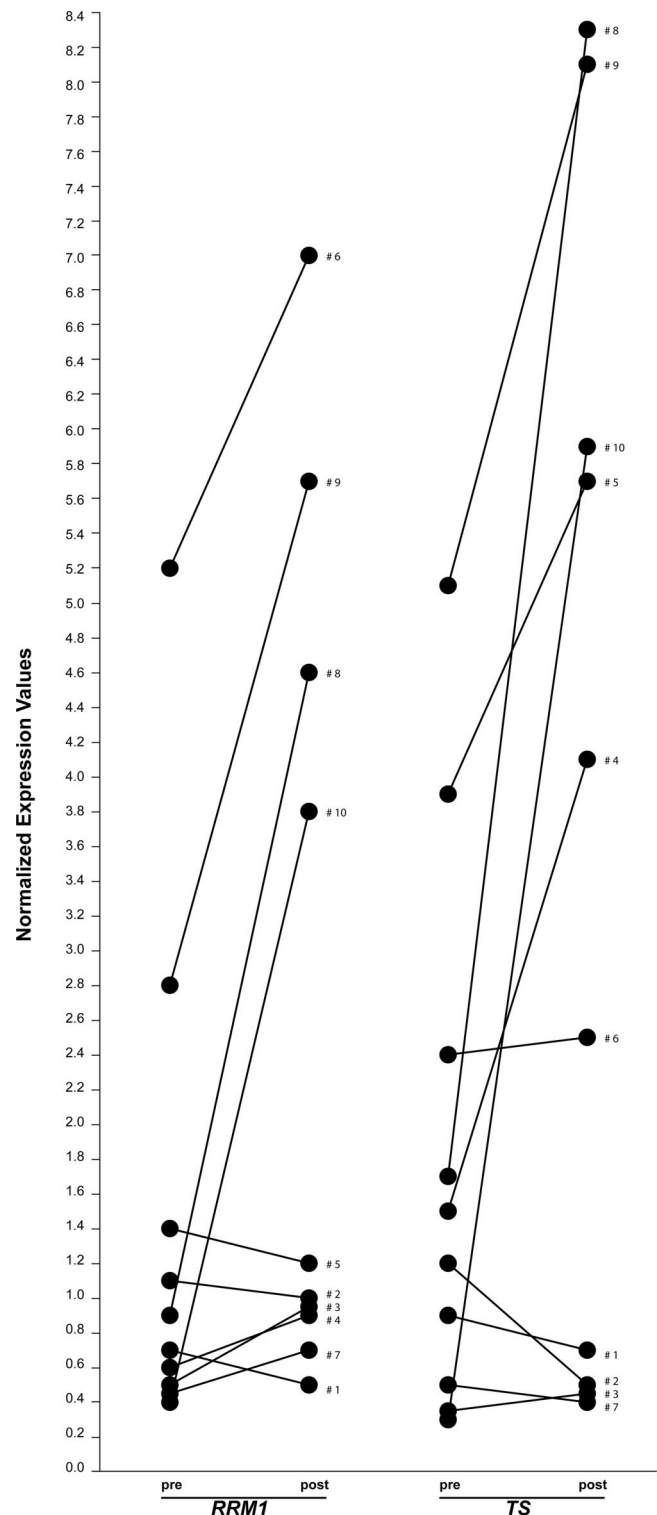


FIGURE 3. Pretreatment and posttreatment mRNA levels of the genes *RRM1* and *TS* in 10 patients.

three with incomplete and two without resection), 14 had died of lung cancer (10 with recurrence, one with incomplete and three without resection), and six had died of causes other than

TABLE 3. Median Overall and Disease-Free Survival by *RRM1* and *TS* mRNA Expression

	Median OS	Log-rank <i>p</i>	Median DFS	Log-rank <i>p</i>
<i>RRM1</i> high (>1.68)	>27.8 mo	0.083	>20.7 mo	0.146
<i>RRM1</i> low (<1.68)	>27.4 mo		21.0 mo	
<i>TS</i> high (>3.39)	>27.8 mo	0.272	>20.7 mo	0.187
<i>TS</i> low (<3.39)	>27.0 mo		21.0 mo	

lung cancer (four from cardiovascular disease). The median follow-up for the 32 living patients was 36.5 months, and it was 30.7 months for the 20 patients with a complete resection and no event. In the 17 patients with disease recurrence after a complete resection, the first site of recurrence was within the region of resected disease in two patients, and it was in previously uninvolved hilar or mediastinal lymph nodes in four patients and in distant sites in 11 patients (five brain, three bone, two lung, one adrenal). In one patient with local recurrence, a resection was successful; however, the disease recurred in the brain.

The median OS was more than 27.8 months, and the median DFS for patients with a complete surgical resection was 33.7 months (Figure 2). The 12-month and 24-month OS rates were 84.6% (95% CI 71.6–92.0%) and 71.0% (95% CI 56.5–81.4%), and the corresponding DFS rates were 67.5% (95% CI 50.7–79.7%) and 51.3% (95% CI 34.6–66.2%), respectively. The radiographic or pathologic response to chemotherapy was not significantly associated with OS or DFS (Table 2).

Pharmacogenomic Variables Predictive of Disease Response

Pretreatment tumor specimens of sufficient quantity and quality for gene expression analysis by real-time reverse transcription polymerase chain reaction were available on 10 and posttreatment specimens on 35 patients. We evaluated if GP therapy would alter the mRNA levels of *RRM1* and *TS*. There was a significant correlation between pretreatment and posttreatment levels for both genes (Spearman's $\rho = 0.786$, $p = 0.025$) suggesting that postchemotherapy gene expression levels are representative of pretreatment levels. However, we observed that gene expression levels seem to increase with treatment (Figure 3). All subsequent analyses were performed on the 35 posttreatment specimens. The ranges of expression and other marker characteristics for all 14 genes are summarized in Table 1. The mRNA levels of *RRM1* were significantly correlated with disease response ($\rho = 0.649$, $p = <0.001$); i.e., low levels were predictive of tumor size reduction and high levels of tumor growth (Figure 1B). Of the 18 patients with *RRM1* expression equal to or below the median of 1.68, 10 had a PR or CR, whereas only 2 of the 17 with high *RRM1* expression responded. The mRNA levels of *TS* were likewise correlated with disease response ($\rho = 0.454$, $p = 0.006$) (Figure 1C); however, after using a Bonferroni adjustment for multiplicity of data analysis, the p value was above the level of 0.0036. Of the 18 patients with *TS* expression equal to or below the median of 3.40, seven had a PR or CR, whereas 5 of the 17 with high *TS* expression responded. The expression levels of all other

genes were not significantly correlated with disease response (Figures 1D, E).

There was no significant difference in OS or DFS between patients with high versus low *RRM1* or *TS* mRNA expression when dichotomized by the gene expression medians of 1.68 for *RRM1* or 3.39 for *TS* (Table 3).

DISCUSSION

A major goal of current research efforts in NSCLC is to increase the efficacy of perioperative systemic therapy in patients with a complete surgical resection through incorporation of molecular parameters into clinical therapeutic decisions. Although most of the data demonstrating efficacy of perioperative systemic therapy has been generated with postoperative treatments, there is presently no evidence to suggest that preoperative therapy is less efficacious. Assuming that both approaches result in similar clinical outcomes, preoperative systemic therapy is substantially better suited for the prospective evaluation of the increasing number of molecular markers on treatment effects. In particular, drug targets can be studied pretherapy and posttherapy, disease response can be used as the primary outcomes variable, and dynamic parameters that may influence drug efficacy can be studied.

We investigated the preoperative efficacy of a nonplatinum doublet, namely GP, in patients with surgically resectable NSCLC. The rationale for this combination was that both agents are antimetabolites with relatively well known mechanisms of action, both are well tolerated, both are efficacious and already integrated into patient care, and prior in vitro studies had suggested cytotoxic synergy between them. Platinum-based postoperative chemotherapy improves 5-year survival by 5 to 15% in patients with completely resected stage II and III disease.^{1–3} However, there is substantial toxicity associated with this therapy,^{1–3} and therapeutic benefit seems to be restricted to patients with low tumoral excision repair cross-complimentary group 1 (ERCC1) levels.¹⁷ Prior randomized phase III neoadjuvant studies that used platinum-based chemotherapy had reported response rates of 33%,⁵ 41%,⁷ 49%,⁶ and 64%.⁴ Approximately 75% of patients received all planned chemotherapy and the chemotherapy-related mortality was approximately 2%. These response rates seem higher than our response rate of 35%; however, 18/52 patients in our study had stage III disease, whereas two of the referenced trials did not include stage III patients,^{5,7} and the proportion of stage III patients in the other two trials was 7%⁶ and 47%,⁴ respectively. In two earlier randomized neoadjuvant trials with platinum-based therapy in patients with stage III disease, the reported response rates were 53% (16/30)¹⁸ and 35% (9/26).¹⁹ We had a priori set our

expected response rate at 50%; a goal, which we did not achieve. We therefore conclude that the combination of GP is unlikely to be superior to platinum-containing doublets if given to an unselected group of patients with resectable NSCLC.

Eighty-seven percent (45/52) of patients in our trial received the planned four cycles of therapy. Five patients had less than four cycles because of treatment-related grade 3 toxicities, and of these, four had surgery with a complete resection. One patient died before surgery from a presumed viral pneumonitis. We conclude that the combination of GP is well tolerated and is deliverable as four biweekly treatments to a proportion of patients that is at least equal to the proportion of patients that can receive a platinum-containing doublet.

Our pharmacogenomic studies indicate that the magnitude of tumor response to GP is associated with tumoral expression of the genes *RRM1* and *TS*. Patients whose tumors express these genes at low levels are more likely to experience a reduction in tumor size compared with those with high levels of gene expression. This association was significant for the gene *RRM1*, but failed to reach the significance level of 0.0036 for *TS*. Because we evaluated a total of 14 genes in these patients, we adjusted the significance level according to Bonferroni. This is a conservative approach to adjustment for multiplicity of data analysis, and most correlative investigations are conducted under less stringent conditions. It is thus our opinion, that the correlation coefficient of (–) 0.454 seen for the association between *TS* expression and response to treatment is remarkable and requires further exploration.

In summary, the clinical correlative investigations described extend the previously known relationship between *RRM1* expression and gemcitabine efficacy to the combination of GP. They also provide reasonable evidence for an association between *TS* expression and efficacy of this combination. In an unselected group of patients with resectable NSCLC, GP provide an objective radiographic response rate of 35% with good tolerability. We conclude that further investigation of this drug combination is warranted with selection of patients based on the expression of *RRM1* and *TS*.

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